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Protein Interactions and Metabolic Response to Stimulating Agents in Isolated Cerebral Tissues: Histones as Inhibitors

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After keeping slices of cerebral tissues in buffered glucose media at 0° for 5–17 hr., their normal increase in respiration in response to electrical pulses is largely lost; on examining a variety of naturally occurring materials for ability to restore the response, fractions of blood plasma were found most active (Marks & McIlwain, 1959). The restoring agent appeared to be protein in nature and fraction IV-4 of Cohn *et al.* (1946), which contained α- and β-globulins, was most active.

The present investigation began with the supposition that fraction IV-4 might interact at a chemical level with a constituent of cerebral tissues. Though the possible types of interaction are very wide, it was decided first to see whether fraction IV-4 gave an obvious change, as of light absorption or precipitation, with material extracted from cerebral tissues. A material which forms a precipitate with fraction IV-4 has been found in cerebral extracts; it appears to be a histone. Moreover, the cerebral extracts which give the precipitates, and also basic proteins from other sources, have been found to be inhibitors of the response of cerebral tissues to stimulating agents.

These findings enable mechanisms to be suggested for the loss of excitability on keeping the tissue at 0°, and for its restoration, and these mechanisms are explored further below.

EXPERIMENTAL

Tissue metabolism. The procedures described by Marks & McIlwain (1959) were followed in preparing tissues from the cerebral cortex of guinea pigs, in applying electrical pulses and high concentrations of potassium salts, and in measuring respiration and lactic acid formation. Tissues were incubated at 37·5° in an oxygenated medium containing (mM): NaCl, 134; KCl, 5·4; $CaCl_2$, 2·6; $MgSO_4$, 1·3; KH_2PO_4, 1·2; glucose, 10 and glycylglycine 30, and adjusted to pH 7·4 with NaOH. This is termed the glycylglycine medium; additions to it are noted in individual experiments below.

Extracts of cerebral tissues. Centrifuging was carried out at 0° with either an MSE machine or Spinco model L ultracentrifuge. Extracts (*a*) and (*b*) were made with cold medium as described by Marks & McIlwain (1959) but buffered with glycylglycine, and extract (*c*) was the 'hot extract' of Marks & McIlwain (1959), made with water.

For extracts (*d*) to (*i*), guinea pigs were killed, the whole brain (3 g.) was removed quickly and put into an ice-cooled homogenizer tube, weighed, 6 ml. of 0·9% NaCl was added and the mixture was ground vigorously for 30 sec. Coarse structures were removed by running the mixture through glass wool, and 15 min. later the filtrate was centrifuged for 10 min. at 10 000 *g*, giving a supernatant (*d*). The precipitate was suspended in 12 ml. of water at 0° and after 30 min. centrifuged as before, giving a supernatant (*e*) and a residue which was resuspended as before; centrifuging for 30 min. then gave not a clear solution but a persistent sol (*f*) as supernatant; the remaining precipitate (*g*) was suspended in glycylglycine medium for test.

After preparing extract (*d*) from other guinea pigs, the residue (from 6·6 g. of brain) was suspended in 20 ml. of 0·2 N-H_2SO_4 and left at 0° for 60 min., centrifuged for 10 min. at 2000 *g* and the residue was extracted as before. The combined supernatants were tied in Visking cellulose tubing and rotated mechanically in 2 l. of water, renewed at intervals of 1½ hr. for 4½ hr., yielding (*h*), the non-dialysable material. This, already neutral, was evaporated at 10 mm. Hg to a few millilitres for test. Extract (*i*) was prepared in the same way as extract (*h*), but commencing with the residue from extract (*d*) from 7 g. of brain, which by the addition of 5 M-NaCl and water was made to 14 ml., 2·6 M in

NaCl; 28 ml. of ethanol was added, and after 60 min. the mixture was filtered and the filtrate dialysed and evaporated as described before.

For extracts (*j*) to (*m*), guinea-pig brain was ground as for (*d*), but in 0·25 M-sucrose containing 0·5 mM-ethylenediaminetetra-acetic acid (EDTA), and fractions were prepared by differential centrifuging according to the methods of Heald (1959) based on those of Brody & Bain (1952) and McMurray, Berry & Rossiter (1957). Extract (*j*) was derived from material sedimenting between 1500 and 104 000 *g*, and extract (*k*) from material sedimenting at 1500 *g*, in each case according to the method used in making extract (*i*) described above. The NaCl yielded an extremely viscous, glairy or mucus-like sludge with the material sedimenting at 1500 *g*, subsequently with ethanol giving a clear solution and a precipitate in sticky shreds, easily filtered through a fluted paper. For the other extracts, the 'crude nuclear' fraction used in making extract (*k*) was further separated by centrifuging above M-sucrose. This fraction, prepared from 5 g. of guinea-pig brain and suspended in 10 ml. of 0·25 M-sucrose with 0·5 mM-EDTA, was carefully run above 20 ml. of M-sucrose in tubes for the Spinco head no. 25, with minimal disturbance of the solution. After 1 hr. at 90 000 *g* this yielded a lighter fraction, between the two layers of sucrose, containing most of the free nuclei and a heavier layer below the M-sucrose. The two portions were placed in separate tubes and suspended in 4 ml. of 0·2 N-H_2SO_4, and after 30 min. at 0° the tubes were centrifuged (5000 *g*) for 10 min. The supernatants were kept and extraction of the residues was repeated until the extracts no longer formed a precipitate or opalescence with a saturated aqueous solution of picric acid. This required three repetitions with the lighter material, yielding (combined) extract (*l*), and one with the heavier fraction, yielding extract (*m*). The extracts were dialysed and evaporated as was extract (*h*). During the dialysis some insoluble material separated, as in comparable preparations of Smillie, Marks & Butler (1955), but tests with picric acid showed the bulk of the material precipitating with this reagent to remain in the solution after dialysis.

Other materials. We are indebted to Mr D. Purdie of Armour Laboratories, Eastbourne, for supplies of ox-blood plasma proteins prepared by ethanol precipitation at controlled pH, according to Cohn *et al.* (1946). Globin was kindly given by Wellcome Laboratories. Histone from calf-thymus nuclei was obtained from L. Light and Co. Ltd. (specimen I) and Nutritional Biochemicals Corp. (specimen II), and protamines (salmine and clupein) were from L. Light and Co. Ltd. (specimen I of salmine) and from British Drug Houses Ltd. (specimen II).

Properties of the extracts. Dry weight was determined after heating samples for 2 hr. at 100°, absence of further change being confirmed after a further 1 hr. at 100°. For observation of the reaction with blood-plasma fraction IV-4, this was shaken with glycylglycine medium (50 mg./ml.) for 1 hr., centrifuged at 2000 *g* and the supernatant used. The tissue extract (0·1 ml.), with or without an equal volume of medium, was placed in a 2 ml. tube with a pointed end, and the IV-4 solution added down the side of the tube in 10 μl. portions until up to 50 μl. had been added, an interval of 3–4 min. being allowed between additions. Precipitates which were formed were often at interfaces, and in some cases dissolved in excess of IV-4; visual impressions of their maximal extent were recorded.

Reaction with picric acid was sought by adding to 0·1 ml. of extract, in tubes as described above, 0·1 ml. of a saturated aqueous solution of picric acid. In some cases (indicated) an additional 0·1 ml. of the extract was taken and N-acetic acid added until no more precipitate formed; the tube was then centrifuged and the picric acid solution added.

RESULTS

Properties of cerebral extracts

The cerebral extracts first prepared were (*a*)–(*h*) of Table 1. When examined at concentrations of a few mg./ml., two extracts gave a slight opalescence with the blood-plasma fraction IV-4; these extracts had been made by allowing the material to stand in glycylglycine medium, and by boiling in water. Only extract (*h*), made by extracting with H_2SO_4 material which was insoluble in iso-osmotic NaCl, gave an appreciable precipitate with fraction IV-4. All eight extracts were then examined for action on the respiration of cerebral tissues, both in a normal, unstimulated condition and also when stimulated by applied electrical pulses. Extracts made in cold medium, in hot water or by grinding in iso-osmotic NaCl markedly accelerated the respiration of the tissue under normal conditions; respiration with pulses was also in some cases accelerated. The extract made with 0·2 N-H_2SO_4 was again unusual in lowering the respiration of the electrically stimulated tissue; it did not have its greatest effect immediately, but after pulses had been applied for 20–30 min.

The method of preparing extract (*h*) indicated that the materials reacting with fraction IV-4 and inhibiting the tissue's response were not easily dialysable and were not extracted from the tissue by iso-osmotic salts or by water, but were extracted by acid. Basic lipids or proteins appeared likely to be present; an alternative method which extracted basic proteins from other tissues was therefore applied, 2·5–3·0 M-NaCl and ethanol being used (see Mirsky, 1943; Butler, Davidson, James & Shooter, 1954; Crampton, Lipshitz & Chargaff, 1954; Crampton, Stein & Moore, 1957; Smillie *et al.* 1955). This yielded extract (*i*), which again formed a precipitate with fraction IV-4 and caused delayed inhibition of the respiration of electrically stimulated tissues (Table 1).

The association of basic proteins with cell nuclei being known, cerebral tissues were next ground and differentially centrifuged before extraction (Table 2). By application of the NaCl–ethanol extraction to the different particulate fractions it was evident that the nuclear fractions differed from others in their swelling with 2·5–3·0 M-NaCl. They yielded material reacting with

fraction IV-4, and again inhibited the electrically stimulated respiration of cerebral tissues, the inhibition increasing with time. The histone-like material could also be extracted from nuclear fractions by 0·2N-H_2SO_4. When samples of the extracts (*a*)–(*m*) were acidified with acetic acid, centrifuged and picric acid was added to the supernatant solutions (see Experimental section), heavy precipitates were given with extracts (*h*), (*i*), (*k*), (*l*) and (*m*), which reacted with fraction IV-4, and also with (*a*) and (*b*); lesser precipitates were obtained with (*d*), (*e*) and (*j*) and none with (*c*) and (*f*).

Table 1. *Properties of cerebral extracts*

Reaction with fraction IV-4 was carried out as described in the Experimental section. In examining action on tissue respiration, the extracts were present initially in 3·5 ml. of glycylglycine medium, the tissue was added and respiration measured during 40 min. at 37° without applied pulses. Condenser pulses of peak potential 10v and time constant 0·4 msec. were then applied at 100/sec. and respiration was measured for a further 40 to 60 min. Values are the means of duplicate or triplicate determinations agreeing to within 6%, except where standard deviation and (in parentheses) number of values are quoted.

Extract (see Experimental section)	Prepared from tissue	Reaction with fraction IV-4 of ox-plasma proteins	Effect on respiration: Dry wt. of extract added (mg.)	Fresh wt. of brain from which extract was derived (mg.)	Respiratory rate: Before pulses	Respiratory rate: With pulses
					(μmoles of O_2/g./hr.)	
None	—	—	—	—	62±2 (6)	120±5 (6)
(*a*)	Cut; leached cold	Slight opalescence	5*†	250	83	112
(*b*)	Cut; leached cold	None	9·8*†	250	96	122
(*c*)	Cut; boiled	Slight opalescence	6·5*	250	80	138
(*d*)	Ground; supernatant	None	8·6*	400	73	121
(*e*)	Ground; water extract	None	8·8	400	58	125
(*f*)	Ground; soluble	None	12·3	500	64	128
(*g*)	Ground; insoluble	None	22·2	500	63	119
(*h*)	Ground; H_2SO_4	Precipitate	19·0	500	59	96, 80‡
			5·7	150	60	106, 84‡
(*i*)	Ground; NaCl–ethanol	Precipitate	18	920	68	118, 86‡
			7·2	370	65	128, 92‡
			1·8	92	65	120, 110‡

* These extracts were examined also at concentrations one-third and one-fifth of those quoted, but were without action on respiration.

† Corrected for weight of constituents of medium used in extraction.

‡ Rates during the second 20–30 min. of application of pulses; when a second rate is not noted in this column, respiration did not change.

Table 2. *Properties of extracts from particulate fractions obtained by centrifuging ground guinea-pig brain*

Extracts were prepared as described in the Experimental section, and tested and recorded as described in Table 1.

Expt.	Fraction	Extract and method of preparation	Reaction with fraction IV-4	Effect on respiration: Dry wt. of extract added (mg./ml. of medium)	Fresh wt. of brain from which extract was derived (mg./ml. of medium)	Respiratory rate (μmoles of O_2/g./hr.): Before pulses	With pulses: First 20–30 min.	With pulses: Second 20–30 min.
1	Fine particles	(*j*) NaCl–ethanol	None	2·9	215	64	121	116
				1·0	86	61	118	116
1	Nuclear fraction	(*k*) NaCl–ethanol	Precipitate	3·1	215	63	113	83
				1·2	86	62	122	104
2	Light nuclear fraction	(*l*) H_2SO_4	Precipitate	2·2	500	56	102	62
				0·7	167	59	100	70
2	Heavier nuclear fraction	(*m*) H_2SO_4	Precipitate less than from (*l*)	1·5	500	59	100	82
				0·5	167	60	104	92
1 and 2	None	—	—	—	—	62±2 (4)	120±4 (4)	113±4 (4)

Basic proteins as inhibitors

The relationships just described were explored by examining the effects of a number of other protein preparations on the metabolism of isolated cerebral tissues (Table 3). Histones from calf-thymus nuclei readily reproduced the inhibition of respiratory response found in cerebral preparations. They were active at concentrations as low as 0·1 mg./ml., and their effect again increased during the application of pulses (Table 3). Much greater concentrations (2·5 mg./ml.) were without action on the tissue's normal respiration; the action on the stimulated tissue extended also to glycolysis, for the additional lactic acid which accumulated when pulses were applied was less in the presence of the histone.

Protamine preparations proved even more active than histones. Clupein and different salmine preparations showed the same selective inhibition of electrically stimulated respiration and glycolysis at concentrations between 10 and 500 μg./ml. Moreover, a protamine preparation also inhibited respiration and glycolysis when these were stimulated by high concentrations of potassium salts, a situation showing many analogies to electrical

Table 3. *Histone and protamine preparations as inhibitors of metabolic changes induced by electrical pulses*

The substances examined were added to glycylglycine medium before the tissue was placed in it. Respiration was measured, electrical pulses were applied and results recorded, as described in Table 1. At the end of the experiment samples of medium were taken for determination of lactic acid. Histone and protamine preparations: see Experimental section.

Substances added and concn. (mg./ml.)		Respiratory rate (μmoles of O_2/g./hr.) Before pulses	With pulses	Lactic acid (μmoles/g./expt.)
None		57	(No pulses)	41±4 (4)
None		58±2 (8)	117±3 (8)	90±5 (4)
Histone I	0·5	61	78, 56*	67
	0·33	60	85, 69*	—
	0·1	58	86, 72*	71
Histone II	0·33	60	70, 55*	63
	0·15	62	107, 90*	—
	0·03	62	120, 110*	—
Salmine I	0·5	56	35	51
	0·1	59±1 (5)	67±4 (5)	48
	0·03	57	84	60
	0·01	56	103	69
Salmine II	0·5	56	54	43
	0·1	57	54	57
Clupein	0·5	58	39	—
	0·1	58	53, 48*	—
	0·05	58	95	—
	0·01	58	114	—
None		62	(No pulses)	42
None		63	112†	56
Clupein	0·1	62	73†	37
Fibrinogen	0·5	58	126	—
	0·1	60	113	—
Gliadin	1·0	58	119	90
	0·5	58	136	—
	0·2	63	131	84
	0·1	58	119	—
Glutenin	1·0‡	60	112	79
	0·2	60	117	84
Globin	1·5‡	62	110	70
	0·3	61	120	99
Plasma albumin	3·0	60	118	83
	0·6	62	119	—

* This value refers to the final 20–30 min. of the experiment and is given when the rate at that time differs from the rate (quoted first) when pulses were first applied.

† No pulses were applied but KCl giving 50 mM-K^+ ions was added from a side arm.

‡ The glutenin specimen was dissolved at an alkaline pH and globin at an acid pH, but at these concentrations partial precipitation took place in the neutral experimental media.

stimulation of the cerebral tissues (McIlwain, 1952; Wollenberger, 1955). Many other proteins were without action on respiration and glycolysis of isolated cerebral tissues, with or without stimulating agents, and when examined in concentrations up to 100 times those in which the protamines acted (Table 3).

Time relationships. The protamine and histone preparations were effective as inhibitors only after they had been in contact with cerebral tissues for some time. In the experiments of Table 3, tissues were incubated with the proteins for a total time of 50 min. (during 40 min. of which respiration was measured) before pulses were applied. When pulses were applied after only 15 min. incubation of tissue with a protamine preparation, the action of the protamine was at first smaller but increased with continued incubation in the same fluid (Table 4). The initial incubation period of 50 min. appeared sufficient to exhibit the maximal action of protamine; it was, however, insufficient for the full action of calf-thymus histone, the effect of which increased between 50 and 120 min. while pulses were applied. This phenomenon was shown also by the inhibitory material, probably a histone, prepared from the nuclear fraction of cerebral tissues.

In other experiments of Table 4, tissues were exposed to protamine-containing media for a limited time only. After 15 min., incubation media (some with and some without protamine) were replaced by fresh media, in all cases lacking protamine. When no protamine had been present, replacement led to respiratory rates some 10% below those normal to the tissues. When a protamine-containing medium was replaced by one lacking protamine, marked inhibition of response was found but the inhibition was smaller than that found when incubation continued in protamine-containing media. With pulses applied at 50 min., inhibition remained at about the levels previously seen when pulses were applied at 15 min. Apparently penetration of protamine into the tissue or combination with the tissue took place progressively during the first 50 min. of incubation.

Interactions with plasma-protein fraction IV-4. All the protamine and histone preparations of Table 3 readily yielded precipitates with fraction IV-4 in neutral solutions in water and in the medium employed in tissue metabolism. Precipitation occurred with a range of concentrations of IV-4, which included the 2–10 mg./ml. at which the fraction is effective in restoring respiratory response to cerebral tissues which have lost response through keeping at 0° (see above). When fresh cerebral tissues were examined in a medium to which had been added an inhibitory concentration of protamine together with fraction IV-4 at 10 mg./ml., the tissue's response was at its normal, high, uninhibited value (Table 5). The combination of IV-4 and protamine was presumably sufficiently firm to render ineffective at least 90% of the protamine. Moreover, when protamine in potentially inhibitory concentrations was present together with cerebral tissues for the first 15 min. of such experiments, it was still possible to annul almost completely its effect by subsequent addition of fraction IV-4. Similarly, after tissues had been incubated in histone-containing media for 15 min.,

Table 4. *Time relationships in the action of a protamine preparation*

Experiments were carried out and results expressed as described in Table 1, except as specified below. When medium was replaced, vessels were taken from their manometers; medium was removed by a Pasteur pipette, the vessel and tissue (still in its grid electrode) were rinsed with 1 ml. of glycylglycine medium and this also was removed; 3·5 ml. fresh medium was added and the vessels were re-equilibrated with oxygen. These operations took 15–20 min. with a group of six vessels.

Clupein present initially (mg./ml.)	Course of experiment (min.)	Respiratory rate (μmoles of O_2/g./hr.)		
		Before pulses	First 30 min. with pulses	Second 30 min. with pulses
0	Pulses applied at 15 or 50 min.	59±3 (6)	118±5 (6)	114±3 (6)
0·1	Pulses applied at 15 min.	—	91	61
0·25	Pulses applied at 15 min.	—	62	40
0·1	Pulses applied at 50 min.	58	58	48
0·25	Pulses applied at 50 min.	57	56	48
0	Medium replaced after 15 min. by medium without clupein; pulses applied at 50 min.	55±3 (4)	102±6 (4)	—
0·1	Medium replaced after 15 min. by medium without clupein; pulses applied at 50 min.	52	79	—
0·25	Medium replaced after 15 min. by medium without clupein; pulses applied at 50 min.	58	66	—

the addition of fraction IV-4 allowed a much greater respiratory response (Table 5).

In view of the finding of the previous section that protamines and histones are delayed in coming into action, the experiments just described did not indicate with certainty whether fraction IV-4 was capable of removing the effects of an established association between inhibitor and tissue. To investigate this, the last experiment of Table 5 was carried out. After the 15 min. incubation of tissues with protamine-containing media, these media were replaced with media lacking protamine, but some of which contained fraction IV-4. The tissue with the IV-4 regained most of its ability to respond to pulses.

DISCUSSION

Basic proteins as inhibitors. The histone and protamine preparations are impressively potent as inhibitors of the response of cerebral tissues to stimulating agents. The preparations used are undoubtedly mixtures of different molecular species, several fractions having been obtained from calf-thymus histone and molecular weights between 10 000 and 30 000 suggested for them (Stedman & Stedman, 1951; Phillips, 1958; Luck, Rasmussen, Satake & Tsvetikov, 1958; Mauritzen & Stedman, 1959). Protamines have also been fractionated and molecular weights of about 5000–8000 proposed (Craig, Konigsberg, Stracher & King, 1958; Ross, 1954). Thus the lowest concentrations at which the excited cerebral tissues were markedly affected, of 0·03 mg. of salmine/ml. or 0·1 mg. of calf-thumus histone/ml., correspond to about 2–10 μM. In the present system barbiturates have effects of comparable magnitude at 300 μM, cocaine at 20 μM and chlorpromazine, among the most potent inhibitors, at 5 μM (McIlwain & Greengard, 1957; Bollard & McIlwain, 1959).

Evidence connecting the effects of added histones with the relatively unresponsive state induced in cerebral tissues by standing in cold media is as follows. (1) Of a variety of cerebral extracts, the few which diminished the tissue's response to stimulation were made by methods known to yield histones, and gave reactions of histones. Inhibitory material was obtained from nuclear preparations of cerebral tissues. Nuclear preparations from cerebral tissues have been found to contain histones similar to those obtained from other somatic cells of the body (Mirsky, 1943; Palladin, Rashba & Shtutman, 1951). (2) The blood-plasma protein fraction IV-4, found to restore responsiveness to cerebral tissues after keeping in cold media (Marks & McIlwain, 1959), restored responsiveness also to fresh tissues whose response had been inhibited by protamines and histones. Fraction IV-4 formed precipitates with the protamines and histones, and with the inhibitory cerebral extracts, at the pH and in the media in which its actions as a restoring agent are exhibited. The formation of protein–protamine complexes is markedly dependent on pH (Ross, 1954), exhibiting relationships with the isoelectric points of the components, and the properties of fraction IV-4 as a restoring agent may in part depend on such characteristics. (3) The unresponsive condition was previously found to be induced simply by keeping the tissue cold in a chemically defined medium normally adequate for response. Responsiveness was not restored by addition of material appearing in the medium, nor was loss of

Table 5. *Antagonism of blood-plasma fraction IV-4 to the action of protamine and histone preparations*

Additions at 15 min. were made by tipping from side arms. Replacement of media was carried out as described in Table 4 and after replacement tissues were incubated for 40 min. without applied pulses, and for 40 min. with pulses; other details were according to Table 1.

Additions to medium before incubation (mg./ml.)	Change at 15 min. incubation	Respiratory rate with pulses (μmoles of O_2/g./hr.)
None	None	118
None	Addition of IV-4 (5 mg./ml.)	121
Histone I (0·33)	None	85, 69*
Histone I (0·33)	Addition of IV-4 (5 mg./ml.)	111, 99*
Salmine I (0·1)	None	67±4 (5)
Salmine I (0·1) and IV-4 (10)	None	127
Salmine I (0·1)	Addition of IV-4 (10 mg./ml.)	101
Salmine I (0·1)	Addition of IV-4 (2 mg./ml.)	98
Salmine I (0·1)	Addition of IV-4 (0·4 mg./ml.)	68
None Salmine I (0·15)	Media replaced by fresh portion of medium without salmine	110 60
Salmine I (0·15)	Medium replaced by medium without salmine and with IV-4 (10 mg./ml.)	94

* Rates during the second 20 min. of application of pulses. When a second rate is not quoted in this column, respiration did not change.

responsiveness affected by adding a variety of adsorbents to the cold media with the tissue. This suggests the change to be within the tissue rather than by loss to the medium, or exchange with the medium.

Possible migration of tissue histone. If the tissue's histone is indeed involved in producing the unresponsive state induced by being kept cold, it would appear necessary for the histone to leave its normal site in the cell nuclei, where it is pictured to be in combination with deoxyribonucleic acid. This appears a relatively loose combination, as emphasized by Mirsky (1943) and illustrated by the methods of extraction used above. A salt-like combination is proposed, though with some specificity between particular histone and nucleic acid components (Crampton, 1957); this is, however, based on the properties of the extracted components. In certain abnormal conditions, histological changes have been noted in cerebral tissues which would be consistent with migration of the type now postulated. Nuclei have been observed to stain with increased intensity with basic dyes, and material staining with acid dyes to appear elsewhere in the cell (Cajal, 1909; Wertham & Wertham, 1934). In the present case, the histone must be pictured as reaching a site involved in the tissue's reaction to electrical pulses, and in view of the basic nature of the histone an acidic site appears likely as a target. Major possibilities are thus the anionic sites or carriers postulated in ion-transport mechanisms or the phosphoproteins involved in the phosphate transfers which take place during the tissue's response to electrical pulses (Heald, 1959). Protamines and histones have also been reported as both activators and inhibitors of enzyme reactions (Krebs, 1954; Madsen & Cori, 1954; Bernfeld, Bernfeld, Nisselbaum & Fishman, 1954).

It must in addition be supposed that the protamines and histones reach such sites when added to media containing normal cerebral tissues. Protamines and histones have been concluded to enter cells of a variety of types (Fischer & Wagner, 1954), and time relationships now observed in the action of the protamines and histones presumably indicate slow penetration of the tissue or its cells. It was noted previously (Marks & McIlwain, 1959) that for cerebral tissues to become unresponsive on keeping at 0° for a few hours some addition was required of the fluid medium normally used in incubation. A relatively small volume, no more than that of the tissue itself, sufficed. Cerebral tissues in such fluids *in vitro* swell under adverse conditions. Absorption of up to two-thirds of their volume of fluid can occur, the swelling being partly intracellular and partly extracellular (Pappius & Elliott, 1956). Possibly the additional fluid or some resulting disorganization gives additional opportunity for migration of substances such as the histone; more direct evidence for this is being sought. Instances of migration of proteins from tissue slices to surrounding fluids, or from one intracellular site to another, have been reported (Stern, Eggleston, Hems & Krebs, 1949; Adams & Burgess, 1959).

SUMMARY

1. Extracts made from cerebral tissues by a variety of methods were examined for action on respiration of guinea-pig cerebral cortex incubated in glucose-containing media. An extract made with acid was found to contain non-dialysable material which inhibited the tissue's response to applied electrical pulses, though having little effect on respiration in the absence of pulses.

2. It was concluded that the inhibitory material is probably a histone. Similar material was obtained by sodium chloride–ethanol extraction, and by extraction of nuclear fractions of the tissue. Histone and protamine preparations from other sources were also inhibitory, being active when incorporated in media at about 5 μM. They inhibited also the tissue's glycolytic response to pulses, as well as its glycolytic and respiratory responses to 50 mM-potassium salts.

3. The blood-plasma fraction IV-4 which restored response to cerebral tissues after they had been rendered unresponsive by being kept at 0° restored response also to tissues treated with protamine and histone. Fraction IV-4 formed precipitates with the basic proteins, and also with the inhibitory cerebral extracts, but not with a number of non-inhibitory cerebral extracts.

4. Time factors in the action of the protamine and histone preparations were investigated. It is suggested that the tissue's histone is involved in the loss of excitability which occurs when cerebral tissues are kept in cold media.

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The Isolation of Lombricine and its Possible Biological Precursor

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In connexion with experiments on the chemical and biological synthesis of 2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate (lombricine) it was desirable to have, as a reference compound, adequate amounts of the naturally occurring material. The isolation of lombricine from the earthworm (*Lumbricus terrestris*) has been described by Van Thoai & Robin (1954), but the low yield reported stimulated a search for an improved method. The simplified procedure described in this paper results in much greater yields of analytically pure material. In addition, experiments are described on the detection and characterization of 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate which, it is suggested, is the immediate precursor of lombricine.

MATERIALS AND METHODS

All reagents used were analytical-grade and solvents used for chromatography were purified by fractional distillation. The reference compounds, 2-guanidinoethyl phosphate, 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate (serine ethanolamine phosphodiester, SEP) and lombricine, were synthesized by D. I. Magrath & I. M. Beatty (unpublished work), who kindly provided samples for chromatographic purposes. Those containing serine were synthesized from the L-isomer. All other compounds were commercial preparations. The reference standards used were chromatographically homogeneous in all solvent systems employed.

The earthworms used were collected in the Canberra area and were of mixed species but were predominantly *Allolobophora caliginosa* and *Octolasium cyaneum*.

The method described by Rosenberg, Ennor & Morrison (1956) for the estimation of arginine was used to determine the concentration of guanidino compounds present in earthworm extracts. These compounds will be referred to as 'guanidine-reacting material'.

Chromatography. All exploratory chromatograms were run in ascending fashion on washed no. 3 Whatman paper, 28 cm. × 28 cm., unless specifically mentioned. Washing was carried out by immersing the ends of the papers in a tank of water and allowing the water to run off for several hours after the front had reached the bottom edges. Two-dimensional chromatograms involving reinforcement with reference compounds were run on pairs of papers cut from one sheet, and care was taken to develop each in the same sequence of solvent systems and in the same direction relative to the original sheet. This was found to produce identical patterns in any one pair of solvent systems.

When chromatography was employed for preparative purposes, solutions of the unknown compounds were applied to the origin as 3–5 mm. wide bands on sheets 60 cm. × 60 cm. The requisite band was located with the aid of markers, cut out and eluted by the method of Reith (1957). This enabled elution from a band approx. 200 cm.^{2} in area in a volume of about 1 ml.